THE GENETIC CONTROL OF SEXUAL REPRODUCTION IN *EUCALYPTUS GLOBULUS*

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INTRODUCTION

While clonal propagation is used to establish some plantations of *Eucalyptus globulus* in countries such as Chile and Portugal, the majority of plantations worldwide are established from seed (Tibbits *et al*. 1997; Griffin 2001). The genetically improved seed used in industrial plantations is usually derived from open pollinated seed orchards (seedling or grafted), but with the advent of single-visit (Williams *et al.* 1999) and one-stop (Harbard *et al.* 1999) pollination procedures an increasing component is now derived from large-scale controlled pollination programs producing elite polymix or full-sib families. Regardless of the system, seed production varies markedly between trees and in most cases the choice of female can strongly influence the profitability of seed production (Leal and Cotterill 1997). In addition, as *E. globulus* has a mixed mating system (Griffin *et al*. 1987; Eldridge *et al*. 1993) and selfing results in severe inbreeding depression for growth traits (Hardner and Potts 1995), outcrossing rate is a key factor. Outcrossing rate will affect the genetic quality of seeds obtained from seed orchards and whether predicted additive genetic gains will be realised. One of the main drivers of the outcrossing rate is the level of self-incompatibility of the female tree (Patterson *et al*. 2001), which varies widely in the species (Potts and Savva 1988; Pound *et al.* 2002a,b, 2003).

This paper reports the results of two studies aimed at determining whether there is a genetic basis to variation in traits affecting sexual reproduction in *E. globulus*. The first study examined the genetic basis of variation in the quantity and quality of open pollinated seed from over 800 seed orchard trees of *E. globulus*. The second study examined the genetic basis of the large variation in self-incompatibility observed within the species.

MATERIALS AND METHODS

Genetic control of reproductive output

Seed was obtained by seedEnergy Pty Ltd from an *E. globulus* seed orchard in Mount Gambier, South Australia (Bawdens) containing 156 open pollinated seedlots sampled from native trees from ten sub-races by the CSIRO in 1987. The orchard contained four replicates, but family representation within replicates was variable due to thinning to 809 trees in 1997. In March of the same year, all trees were treated with the flowering stimulator paclobutrazol as a Cultar® soil drench applied at a rate of 0.3 gram of active ingredient per cm of tree diameter. The effect of this treatment was evident the following spring with large numbers of flower bud initials, and led to a large flower bud crop in 1999. In 2000, one year old capsules were harvested from all the trees in the orchard by hand-picking felled trees or using a hoist to access the canopies of standing trees. The capsules harvested were then weighed and a sub-sample of 25 capsules was collected from each tree. These sub-samples were weighed, the capsules were then dried in an oven until the capsule valves were fully open (at 40^0C), and the seed and chaff extracted and weighed. The seedlots were then cleaned with a vacuum separator to separate the viable seed from the chaff and inviable (unfilled) seed. The cleaned seed from replicates 1 and 2 was then sieved into six size classes (>2mm, >1.7mm<2mm, >1.4mm<1.7mm, >1.18mm<1.4mm, >1mm<1.18mm and <1mm). The number of viable seed was then counted in each seedlot by hand or using a Pfeuffer (Contador) seed counter. The total number of capsules harvested per tree was estimated from the total weight of capsules harvested divided by the weight of an individual capsule calculated from the 25 capsule subsample. This value was multiplied by the number of seed per capsule obtained from the subsample to estimate the total number of seeds produced per tree.

The reproductive traits listed in Table 1 were analysed using ASREML (Gilmour 1999) to fit the mixed model:

$y = \mu + \text{sub-race} + \textit{replicate} + \textit{family (sub-race)} + \textit{residual}$

where μ is the population mean, sub-race is the fixed effect of the 10 sub-races (Table 2), *replicate* is the random replicate effect, *family (sub-race)* is the random variation between open pollinated families within sub-race and *residual* is the unexplained random variation. In the case of TOTCAP and TOTSD, an additional fixed term was included in the model to account for

differences in the method of capsule harvest (tree felling or hoist). The narrow-sense heritability (h^2_{op}) was calculated using the family within sub-race variance component divided by a coefficient of relatedness of 0.4 to estimate the additive variance, and dividing by the phenotypic variance estimated from the sum of the family within sub-race and error variance components. Bivariate analyses were also undertaken with ASREML to estimate the correlations between traits at the sub-race ($r_{sub\text{-}race}$) and family within sub-race (r_g) levels, but in this case sub-race was treated as a random term. The significance of the random family within sub-race term and the correlations was tested using a 1 and 2-tailed likelihood ratio test respectively. The fixed sub-race effect was tested with an F test.

Table 1. The traits analysed, the number of individuals and families, the significance of the sub-race ($F_{sub\text{-}race}$) and family within sub-race effects (Z_{family}) and within sub-race narrow-sense heritabilities (h^2± se). Traits with superscripts were transformed ([1]square root, [2]power 0.25), (n.s. not significant, * P<0.05, ** P<0.01, *** P<0.001)

Trait	Description	*n_indiv*	*n_families*	$F_{sub\text{-}race}$		Z_{family}		h^2	s.e.
CAPWT	Mass of a single capsule (g)	809	156	7.6	***	1.3	n.s.	0.08	0.06
NSD_WTSC[1]	Number of seed per gram of seed and chaff	809	156	2.5	*	4.2	***	0.43	0.09
NSD_CAP[1]	Number of seed per capsule	809	156	3.3	***	3.4	***	0.30	0.08
TOTCAP[2]	Total number of capsules per tree	809	156	7.2	***	3.1	***	0.27	0.08
TOTSD[2]	Total number of seed per tree	809	156	7.6	***	3.5	***	0.33	0.08
PRO_ABC	Proportion of seed > 1.4mm	410	143	21.2	***	1.9	*	0.27	0.14

Genetic control of self-incompatibility

Controlled pollinations were undertaken over two flowering seasons in a clonal breeding arboretum in Western Australia in 1997 and 1998. Pollinations were completed on 32 ramets from 14 genets. Six of the genets were tested in both years. The controlled crosses were done using the three-visit procedure. On the first visit to the tree, flower(s) were chosen where the operculum was just starting to lift away from the receptacle. The stamens were then emasculated and the flower(s) isolated with a bag, and the branch tagged. A week later when the stigma was receptive the flowers were pollinated, with either outcross or self pollen. On each ramet, generally ten flowers were crossed with self pollen and ten flowers with outcross pollen, and 10 to 26 flowers were labelled and left to open pollinate. On each ramet, outcrosses were produced using five different male pollens each from a different race of *E. globulus* (Southern Tasmania [Taranna locality], King Island, Furneaux, South-eastern Tasmania [Hobart locality] and Otway Ranges [Western and Eastern combined], see Dutkowski and Potts 1999). Each unique pollen was applied in a separate bag. Different ramets of the same genotype were outcrossed with pollens from the same five races but different genotypes. The isolation bags were then removed four to eight weeks later. A year later the capsules that remained were collected and the seed counted. Self-incompatibility was calculated with the following equation:

$$\%SI = 100 \times \frac{OUTCROSS - SELF}{OUTCROSS}$$

where OUTCROSS is the number of viable seed per flower from outcross pollen, and SELF is the number of viable seed per flower from self pollen (Pound *et al.* 2002a). For genotypes with more than one ramet screened, a mixed model was fitted to the %SI data using Proc MIXED (SAS V8) with genotype fixed and season as a random effect.

RESULTS

Genetic control of reproductive output

All the traits studied showed significant sub-race differentiation, with F-values ranging from 2.5 (P<0.05) for NSD_WTSC to 21.2 (P<0.001) for PRO_ABC (Table 1). Trees from Southern Tasmania had the heaviest capsules, along with other core *E. globulus* sub-races - South-eastern Tasmania and North-eastern Tasmania (Table 2). The sub-races with the lightest capsules were the Strzelecki Ranges and Strzelecki Foothills. The capsule weight was directly correlated with capsule size as measured by either maximum diameter (r=0.93, P<0.001) or height (r=0.85, P<0.001). The number of viable seed per gram of seed plus chaff varied from 0.3 to 355 (mean = 114) across all trees and sub-races means varied from 101 to 160 (Table 2). The lowest number of seed per gram of seed plus chaff was obtained from trees from North-eastern Tasmania, Strezlecki Ranges and Strezlecki Foothills whereas the highest number was obtained from trees from Western Tasmania and Flinders Island. The mean number of viable seed per capsule ranged 0.06 to 80 (mean = 23) and sub-race means ranged from 20 to 37 (Table 2). Neither the number of seed obtained per gram of seed plus chaff nor number of viable seed per capsule were correlated with the size of the capsule as measured by capsule weight (NSD_WTSC $r_{sub\text{-}race}$ = -0.02, P= 1.000; NSD_CAP $r_{sub\text{-}race}$ = 0.08, P= 0.893) at the subrace level. The size of the seed, however, did increase with capsule size as indicated by a significant correlation between the percentage of large (>1.4mm) seed and capsule weight at the

sub-race level ($r_{sub\text{-}race}$ = 0.96, P= 0.018). In this case it was the core Tasmanian sub-races from eastern Tasmania (Southern Tasmania, South-eastern Tasmania, North-eastern Tasmania and Inland North-eastern Tasmania) that had the highest percentage of large seed and the Strezlecki Ranges sub-race which had the lowest percentage (Figure 1). There was no evidence of genetic variation in capsule weight between trees within sub-races (Table 1). However, for the proportion of large seeds as well as the number of seeds per gram of seed plus chaff and per capsule there was significant variation between families within sub-races (Table 1), and the heritability of their variation within sub-races ranged from 0.27 to 0.43.

Table 2. Sub-race least square means for five reproductive traits in *Eucalyptus globulus*. Values correspond to those calculated using untransformed data whereas the significance testing was based on transformed data for four traits (see Table 1). Means with common letters were not significantly different following the Tukey-Kramer adjustment for multiple comparisons

Sub-race	capwt (g)		nsd_wtsc (n seed/g)		nsd_cap		totcap		totsd	
Strzelecki Ranges	4.11	e	101	b	21	b	1360	ce	25,916	c
Strzelecki Foothills	4.10	de	103	ab	20	b	1787	abcde	30,989	cd
Western Otways	4.43	acd	115	ab	24	b	2675	abcd	65,075	abcd
King Island	4.38	abcde	123	ab	30	ab	3473	a	103,845	ab
Western Tasmania	4.36	abcde	160	a	37	a	3442	ab	129,355	a
Southern Tasmania	4.80	a	108	ab	25	ab	1988	abcde	58,273	abcd
South-eastern Tasmania	4.72	ab	111	ab	23	ab	1554	bce	37,561	cd
North-eastern Tasmania	4.59	abc	101	ab	20	b	1732	abcde	33,215	cd
Inland North-eastern Tasmania	4.39	abcde	134	ab	25	ab	1009	e	26,870	c
Flinders Island	4.29	abcde	149	ab	28	ab	3282	abc	69,580	abc

Trees in the seed orchard differed markedly in their fecundity with the estimates of the total number of capsules and seed collected per tree ranging from 86 to 12,309 (mean = 2412) and 69 to 464,761 (mean = 59,240) respectively. There was a genetic basis to this variation in reproductive output with significant differences between sub-races and between families within sub-races for both numbers of capsules and seeds harvested per tree (Table 1). There was a three-fold and five-fold difference in sub-race means for total number of capsules per tree and total number of seed per tree respectively (Table 2). In general, trees from King Island and Western Tasmania were the most fecund (Table 2). The heritability of the within sub-race variation in the number of capsules and seeds collected per tree was 0.27 and 0.33 respectively (Table 1). Within sub-races, genetic variation in the total number of seeds obtained per tree was significantly positively correlated with genetic variation in the total number of capsules harvested (r_g = 0.88, P= 0.007), the number of seed per gram of seed plus chaff (r_g = 0.71, P= 0.004), and the number of seeds per capsule (r_g = 0.65, P= 0.019). However variation between sub-races in this trait was only significantly correlated with variation in the total number of capsules harvested (r_g = 0.98, P= 0.003) and the number of seeds per capsule (r_g = 0.93, P= 0.030). At no level was there a significant genetic association between total number of seed harvested per tree and seed size (PRO_ABC vs TOTSD $r_{sub\text{-}race}$ = -0.08, P=0.88; r_g = -0.31, P= 0.456).

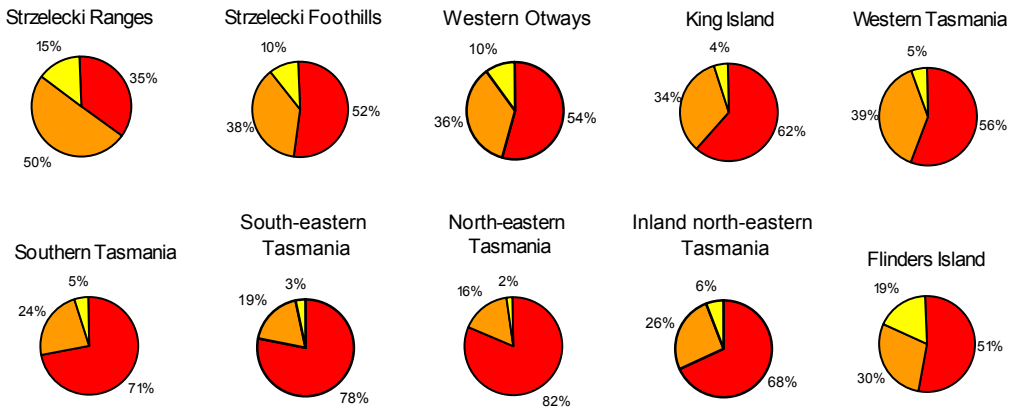

Figure 1. Percentage of each seed size class for each of the ten sub-races. Dark shading is seed >1.4mm, medium is seed >1.18mm<1.4mm and light is seed <1.18mm

Genetic control of self-incompatibility

Seed set was significantly (P<0.001) reduced following self-pollination as compared to outcross pollination, from 12.1 to 1.8 seed/flower and 21 to 3.3 seed/capsule respectively, the difference between these being due to flower loss. Even in comparison with seed set following open pollination (10.2 seed/flower and 15 seed/capsule), the number of seed set after selfing was much lower (Figure 2). There was only a significant difference between outcross and open pollination for the number of seeds per capsule (Figure 2).

Estimates of the level of self-incompatibility (%SI) of individual ramets ranged from –2.4% (fully self-compatible) to 100% (fully self-incompatible), with the overall population SI level estimated to be 85.6%. There was a highly significant ($F_{7,16}$ = 13.92, P<0.001) difference between genotypes in their level of self-incompatibility but no significant effect of season (Z = 0.55, P=0.2928) was detected. Mean self-incompatibility level of the genotypes varied between 14% and 102% (lsmeans of the %SI for the 8 genotypes with multiple ramets were 14, 62, 83, 88, 92, 93, 101 and 102%).

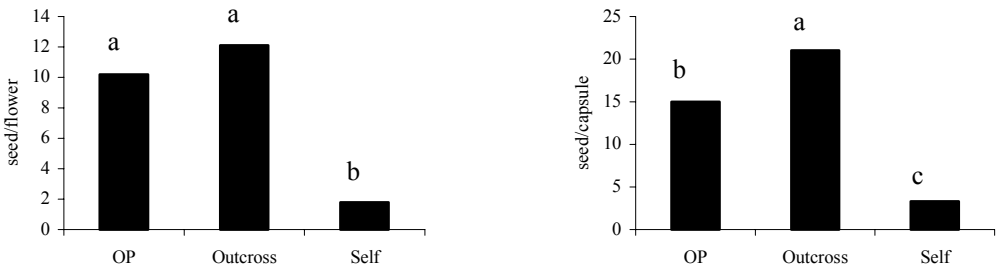

Figure 2. Mean seed set per flower and per capsule after open pollination (OP), outcross and self controlled pollinations

DISCUSSION

This study suggested there is a strong genetic basis to the traits that affect fecundity in *E. globulus*. This could be due to inherent overall variation in fitness, variation in adaptation to the specific experimental site or inherent differences in resource allocation strategies (for example between vegetative versus reproductive structures). Such variation is quite understandable at the sub-race level where previous studies suggest that the sub-races differ genetically for numerous adaptive traits (e.g. Dutkowski and Potts 1999). However, within populations (i.e. sub-races in our case) traits closely linked to fitness, such as total seed output are normally expected to have low heritability (Falconer and MacKay 1996). There is clearly significant genetic diversity within *E. globulus* populations for adaptive traits such as frost, drought and pest resistance and the heritability of growth in *E. globulus* (average h^2_{op} for many studies of dbh is 0.21; Lopez *et al*. 2002) is only slightly lower than those observed for the reproductive traits. Difference in adaptation and resource allocation strategies could therefore be the cause of the genetic variation in reproductive output within sub-races. However, it is also possible that in our case variation in susceptibility to the flowering enhancement effects of paclobutrazol was confounded with natural genetic variation in fecundity. To resolve what is the best

explanation for these large genetic components will require studying the reproductive output of this germplasm at other sites in the absence of paclobutrazol.

Regardless of the cause, these large genetic differences can have a large impact on the cost of seed production, not only by affecting seed output but also the quality of the seed. Seed output and quality will be important for open pollinated seed orchards, while seed/capsule will also be particularly important for control or mass pollinated seed costs. Seed size is an important quality factor and nurseries normally segregate *E. globulus* seed into different seed sizes to promote even germination and seedling growth (Watson *et al.* 2001). This practise has been based on research that has found that larger seeds germinate faster and better, produce larger seedlings and show a higher seedling survival rate (Battaglia 1993; Martins-Corder *et al.* 1998). In many cases very small *E. globulus* seed is discarded. This study has shown that there are large genetic differences both between and within sub-races in seed size and it was the sub-races with the largest capsules which had the highest proportion of large seed. While there was no significant statistical association between total seed output and seed size, the Strzelecki Ranges sub-race was amongst the least fecund and had the lowest proportion of large seed. This sub-race is favoured in plantations and breeding programs of *E. globulus* for pulpwood production and these poor seed attributes would substantially increase the cost of seed production from elite genotypes from this sub-race.

Variation in self incompatibility has been reported in many eucalypt species (Potts and Savva 1988), but this is the first study demonstrating a genetic basis to this variation. This is important as it means there is the possibility for utilising highly self incompatible trees as females for seed collection or crossing. With open pollination this offers a means of increasing outcrossing rate, in the case of crossing there is the opportunity for minimising contamination from self pollination. Further studies are required to test the stability of self incompatibility across seasons and sites. Research is also required to ensure that selection for highly self incompatible trees will not adversely affect outcross progeny performance. Self incompatibility in *E. globulus* is predominantly controlled by late-acting, post-zygotic mechanisms (Pound *et al.* 2002a,b & 2003). It is possible that growth of outcross progenies may be depressed when both parents have a high degree of self incompatibility. This is because the self incompatibility mechanism may depend on the expression of numerous deleterious recessive alleles in the embryos, such that selfed embryos abort when homozygous for several of these alleles (Sedgley 1994). If the deleterious alleles are expressed at later stages in life due to incomplete dominance, the performance of some outcross progenies may be reduced (Fu and Ritland 1994).

In summary, this study has shown that there is strong genetic control of traits affecting sexual reproduction in *E. globulus*. There is a clear potential for exploitation of this variation to reduce the cost of seed production in open pollinated, controlled pollination and mass supplementary pollination systems.

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